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Original Paper

Inhibition of Experimental Gastric Carcinogenesis, Induced by *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine in Rats, by Sodium Nitroprusside, a Nitric Oxide Generator

H. Iishi, M. Tatsuta, M. Baba, R. Yamamoto, H. Uehara and A. Nakaizumi

Department of Gastrointestinal Oncology, Osaka Medical Centre for Cancer and Cardiovascular Diseases, 3-3 Nakamichi 1-chome, Higashinari-ku, Osaka 537, Japan

The effects of prolonged administration of sodium nitroprusside (SNP), a generator of nitric oxide (NO), on gastric carcinogenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and the labelling index of the gastric mucosa were investigated in male Wistar rats. The rats received intraperitoneal injections of 2 or 4 mg/kg body weight of SNP every other day after 25 weeks' oral treatment with the carcinogen. Prolonged administration of SNP at 4 mg/kg body weight, but not at 2 mg/kg body weight, significantly decreased the incidence of gastric cancers in experimental week 52. However, it did not affect the histological types or depths of involvement of gastric cancers. SNP at 4 mg/kg body weight, but not at 2 mg/kg body weight, also significantly decreased the bromodeoxyuridine labelling index of the antral epithelial cells. These findings indicate that SNP inhibits gastric carcinogenesis and suggest that this effect may be related to the suppression of proliferation of the antral epithelial cells.

Key words: gastric carcinogenesis, sodium nitroprusside, nitric oxide, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, chemoprevention
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INTRODUCTION

NITRIC OXIDE (NO) is a potent biological molecule derived from the terminal guanidonitrogen of L-arginine [1, 2] which is catabolised by constitutive or inducible NO synthase expressed in macrophages, endothelial cells, hepatocytes, smooth muscle cells and platelets [3, 4]. NO has biological effects such as vasodilatation [5] and platelet aggregation [6]. It is also a major cytotoxic mediator produced by macrophages [1] and endothelial cells [7] responsible for the destruction of tumour cells.

Sodium nitroprusside (SNP) is a potent hypotensive agent commonly used to control hypertension during surgery and in hypertensive emergencies and to improve cardiac function after myocardial infarction [8, 9]. It yields NO after metabolic activation and inhibits the growth of human neuroblastoma cells in a dose-dependent manner [10]. NO-mediated cytotoxicity has mainly been studied *in vitro* [11], but the role of NO in mediating tumoricidal activity *in vivo* is not well understood. Therefore, in the present study, we examined the effects of long-term intraperitoneal (i.p.) administration of SNP on experimental gastric carcinogenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in rats.

Correspondence to H. Iishi.
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MATERIALS AND METHODS

Animals

Seventy-five inbred male Wistar rats (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in suspended, wire-bottomed metal cages at a controlled temperature (20–22°C) and humidity (30–50%), with a 12 h–12 h light–dark cycle.

Experimental design

The animals were given drinking water containing MNNG (50 μg/ml; Aldrich, Milwaukee, Wisconsin, U.S.A.) and regular food pellets (Nihon Nosan, Yokohama, Japan) for 25 weeks. The MNNG was dissolved in de-ionised water at a concentration of 1 mg/ml and kept in a cool (4°C), dark place. Just before use, the stock solution was diluted to 50 μg/ml with tap water. Each rat was given 40 ml of MNNG solution (less than a single rat can consume in 48 h) from a bottle

covered with aluminium foil to prevent the photolysis of MNNG; the solution was replenished every other day. Each rat consumed 40 ml of MNNG solution within 48 h for 25 weeks from the start of the experiment.

At week 26, the animals were divided randomly into three groups of 25 rats each, after which they were given normal tap water *ad libitum* from an automatic watering system. They were given alternate-day i.p. injections as follows until the end of the experiment in week 52. Group 1, the control group, was given the vehicle, 0.9% NaCl solution; groups 2 and 3 were given 2 mg/kg or 4 mg/kg body weight of SNP, respectively. SNP (Sigma, St Louis, Missouri, U.S.A.) was dissolved in 0.9% NaCl solution. The injections were given in a volume of 2 ml/kg body weight between 1400 and 1500 h each day.

Histological observations

The animals that survived for more than 48 weeks were included in the analysis as the first tumour of the glandular stomach was found in a rat in group 1 that died in week 48. All surviving rats were killed at the end of the experiment in week 52 and examined at autopsy. The stomach was opened along the greater curvature, pinned flat on a cork mat, and fixed with picric acid–formaldehyde solution for histological examination. The fixed stomach was cut into longitudinal strips 3 mm wide. The specimens were embedded in paraffin, and serial sections of 5 μm thickness were stained with haematoxylin and eosin. The sections were examined without knowledge of which group they were from.

Definition and classification of gastric cancers

Histologically, adenocarcinomas were defined as lesions in which neoplastic cells penetrated the muscularis mucosa to invade the submucosa or deeper layers and classified as very well differentiated, well differentiated, or poorly differentiated [12].

Measurement of labelling index

The labelling index of gastric mucosal cells was examined in weeks 30 and 52 in 5 rats from each group with an immunohistochemical analysis kit for bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.) [13, 14]. Briefly, five rats in each group were starved for 12 h and then treated with i.p. injections of 2 ml/kg body weight of 0.9% NaCl solution (group 1) or 2 mg/kg (group 2) or 4 mg/kg (group 3) body weight of SNP in 2 ml/kg body weight of 0.9% NaCl solution. One hour later, they received an i.p. injection of 20 mg/kg body weight of BrdU and were killed with ether 1 h later. The stomach was removed and fixed in 70% ethanol for 4 h. The fixed stomach was cut into longitudinal strips 3 mm wide. The specimens were embedded in paraffin, and sections of 3 μm thickness were immersed in 2 N HCl for 30 min at room temperature and neutralised in 0.1 N $Na_2B_4O_7$, then immersed in methanol containing 0.3% H_2O_2 for 30 min and treated with 10% horse serum. The sections were then stained with anti-BrdU monoclonal antibody (diluted 1:20) for 2 h at room temperature, stained with biotin-conjugated horse antimouse antibody (Vector Laboratories, Burlingame, California, U.S.A.; diluted 1:200) for 30 min, and then treated with avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min. The reaction product was localised with 3,3′-diaminobenzidine tetrahydrochloride. The BrdU-labelled cells were identified by the presence of a dark pigment over their nuclei.

To determine the labelling index of the gastric mucosal cells, BrdU-labelled and -unlabelled cells in the zone of proliferating cells [15] were counted without knowledge of which group the preparations were from. The zone of proliferating cells in the fundic mucosa was defined as a 250 μm rectangle between the highest and lowest labelled cells in a well-oriented section; 10 such rectangular areas per rat were examined. In the antral mucosa, all cells below the highest labelled cell in each gland were regarded as being within the zone of proliferating cells. In this case, 100 well-oriented glands were examined from each rat. From these measurements, the labelling index was calculated as the number of BrdU-labelled cells per total number of cells within the zone of proliferating cells.

Statistical analysis

The results were analysed using the χ^2 test, Fisher's exact probability test, or one-way analysis of variance with Dunn's multiple comparison [16]. Data are given as means ± standard error (SEM). 'Significant' indicates a calculated P value of less than 0.05.

RESULTS

Incidence, number, histological type and depth of involvement of gastric cancers

5 rats in each group were killed in week 30 for measurement of the labelling index of the gastric mucosal cells. 1 rat in group 2 died in week 38, and because tumours were not found in this animal, it was excluded from the analysis. At week 52, there were no significant differences in body weights among the three groups.

The incidence of gastric cancers in group 3 (4 mg/kg of SNP) (6/20, 30%) was significantly less than in the control group 1 (14/20; 70%). Administration of 2 mg/kg of SNP slightly decreased the incidence of gastric cancers compared with that in group 1, but the difference was not of statistical significance ($P = 0.085$). The numbers of gastric cancers per tumour-bearing rat were not significantly different among the three groups (Figure 1).

All gastric cancers were found in the antral mucosa and were histologically adenocarcinomas (Figure 2). There were

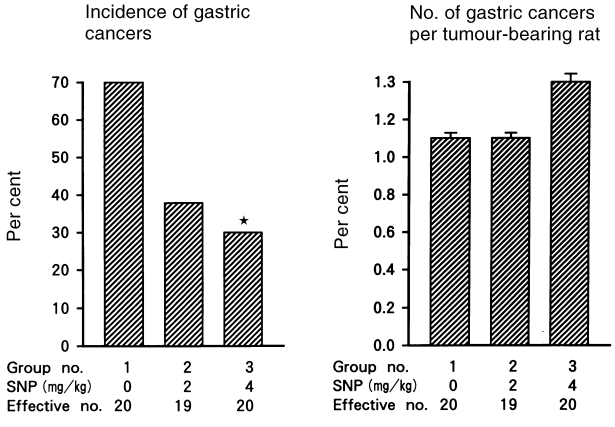

Figure 1. (a) Incidence and (b) number of gastric cancers in MNNG-treated rats. *Significantly different from the value for group 1 at $P < 0.05$.

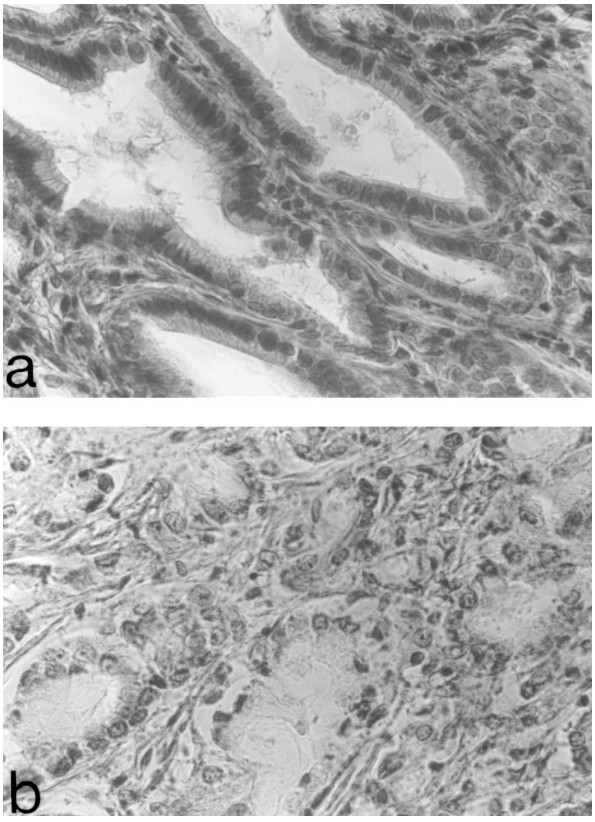

Figure 2. Microphotographs of gastric adenocarcinomas induced in MNNG-treated rats. (a) Very well differentiated adenocarcinoma; (b) well differentiated adenocarcinoma. Haematoxylin and eosin, ×200.

no significant differences in the histological types of adenocarcinomas among the three groups (Figure 3). No poorly differentiated cancers were found in this series. Furthermore, there were no significant differences in the depths of involvement of gastric cancers among the three groups (Figure 3). No overt metastases were seen in any rat.

Labelling index

At weeks 30 and 52, administration of SNP at 4 mg/kg significantly decreased the labelling indices of the antral mucosa compared with group 1 (Figures 4 and 5). At week 52, administration of SNP at 2 mg/kg slightly, but not significantly ($P = 0.085$), decreased the labelling index of the antral mucosa compared with group 1. Administration of SNP did not affect the labelling indices of the fundic mucosa at either time examined.

In this series, the labelling indices of gastric cancers were not measured as there were few tumours found in the rats that were given BrdU.

DISCUSSION

Our present results show that long-term administration of the NO-generating agent SNP at a high dose inhibits gastric carcinogenesis induced by MNNG in rats. The administration of SNP at a low-dose slightly decreased the incidence of gastric cancers. Lee and Wurster [10] also reported that SNP inhibits tumour cell growth in a dose-dependent manner and that this inhibition is blocked by haemoglobin, which has an NO-inhibitory effect. These results imply that the growth-inhibitory action of SNP may be related to NO production.

NO has several actions [17], including inhibition of DNA synthesis [11, 18, 19], mitochondrial respiration [1, 20] and cytochrome P450 [21], and interference with iron-sulphur protein [11]. The exact mechanism by which SNP inhibits gastric carcinogenesis is unknown, but at least three explanations may be considered. One possible explanation is the protective effect of NO against the cytotoxicity of reactive oxygen species. Wink and associates [22, 23] found that NO attenuates the cytotoxicity of superoxide or hydrogen

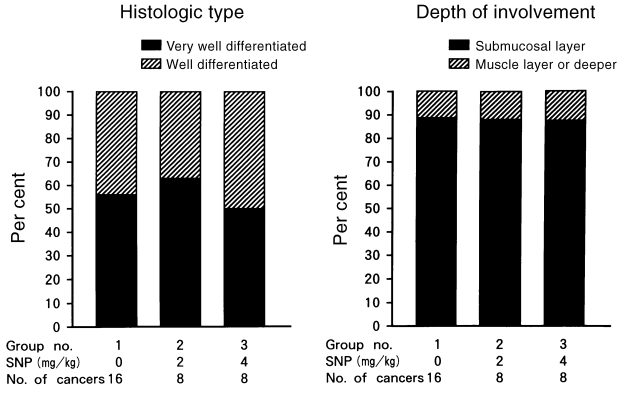

Figure 3. Histological type and depth of involvement of gastric cancers in MNNG-treated rats. There were no significant differences among the three groups.

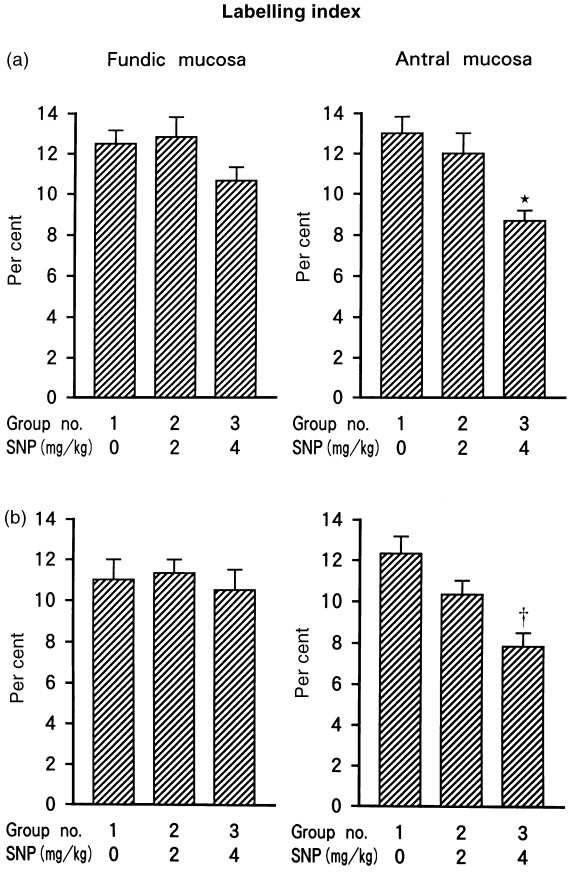

Figure 4. Labelling index of the gastric mucosa in MNNG-treated rats at (a) week 30; (b) week 52. Significantly different from the value for group 1: *$P < 0.05$, †$P < 0.01$.

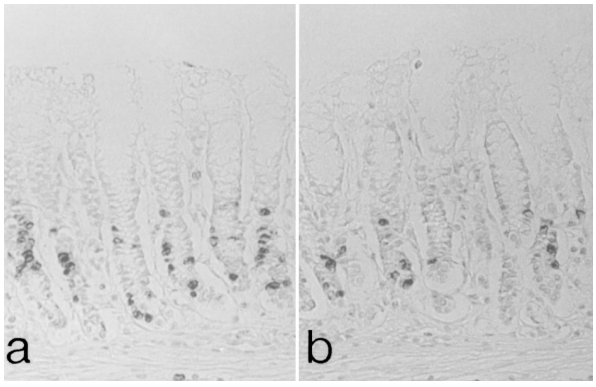

Figure 5. Immunohistochemical photographs of the gastric mucosa at week 52 from (a) a control rat and (b) a rat treated with SNP at 4 mg/kg body weight. BrdU-labelled cells were seen less in (b) than in (a), ×100.

peroxide in Chinese hamster lung fibroblasts (V79 cells). This result suggests that NO provides protection against reactive oxygen species.

A second possible mechanism of inhibition is the action of macrophages in the host immune response against cancer. Hibbs and colleagues [24] reported that the cytotoxicity of macrophages is induced by the amino acid L-arginine and that its effector molecule is NO. Cytotoxic activated macrophages have various effects on tumour cells, including inhibition of mitochondrial respiration and DNA replication [25]. Yim and associates [26] found that treatment with an NO synthase inhibitor increases growth and delays rejection of highly antigenic ultraviolet radiation-induced regressor tumours transplanted in mice. They concluded that macrophage-derived NO might contribute to the antitumor immune response *in vivo*.

A third possible mechanism by which SNP inhibits gastric carcinogenesis is inhibition of DNA synthesis. Nakai and colleagues [27] found that NO and NO-producing compounds, such as SNP and glyceryl trinitrate, inhibit basal and 10% fetal calf serum-induced DNA synthesis in RAC-1 clonal rat aortic smooth muscle cells. Firnhaber and Murphy [28] also found that non-toxic rates of NO generation (0.1–1.0 μM/min) inhibit DNA synthesis in human lung cells and skin fibroblasts. In the present study, we found that long-term administration of SNP significantly decreases the labelling indices of the antral epithelial cells.

In conclusion, we found in the present study that gastric carcinogenesis induced by MNNG is significantly inhibited by SNP. Our findings suggest that this effect may be related to inhibition of cell proliferation in the gastric mucosa.

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